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(54) Title: VECTOR PARTICLES RESISTANT TO INACTIVATION BY HUMAN SERUM

(57) Abstract

A retroviral vector particle resistant to inactivation by human serum. The vector particles preferably include p15E protein wherein at least a portion of the DNA encoding p15E protein is mutated such that the vector particle is resistant to inactivation by human serum. The vector particles may further include a protein containing a receptor binding region which binds to the receptor of a human target cell, thereby enabling the direct introduction of desired heterologous genes *in vivo*, whereby the vector particle including the heterologous gene travels directly to a targeted cell or tissue.

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VECTOR PARTICLES RESISTANT TO INACTIVATION BY HUMAN SERUM

This invention relates to "injectable" vector particles. More particularly, this invention relates to vector particles, such as retroviral vector particles, wherein such vector particles are resistant to inactivation by human serum.

Vector particles are useful agents for introducing gene(s) or DNA (RNA) into a cell, such as a eukaryotic cell. The gene(s) is controlled by an appropriate promoter. Examples of vectors which may be employed to generate vector particles include prokaryotic vectors, such as bacterial vectors; eukaryotic vectors, including fungal vectors such as yeast vectors; and viral vectors such as DNA virus vectors, RNA virus vectors, and retroviral vectors. Retroviruses which have been employed for generating vector particles for introducing genes or DNA (RNA) into a cell include Moloney Murine Leukemia Virus, Spleen Necrosis Virus, Rous Sarcoma Virus and Harvey Sarcoma Virus. The term "introducing" as used herein encompasses a variety of methods of transfering genes or DNA (RNA) into a cell. Such methods include transformation, transduction, transfection, and infection.

Vector particles have been used for introducing DNA (RNA) into cells for gene therapy purposes. In general, such a procedure involves obtaining cells from a patient and using a vector particle to introduce desired DNA (RNA) into the cells and then providing the patient with the engineered cells for a therapeutic purpose. It would be desirable to provide alternative procedures for gene therapy. Such an alternative procedure would involve genetically engineering cells in vivo. In such a procedure, a vector particle which includes the desired DNA (RNA) would be administered to a patient for in vivo delivery to the cells of a patient.

It is therefore an object of the present invention to provide gene therapy by introduction of a vector particle, such as, for example, a retroviral vector particle, into a patient, wherein the vector particle is resistant to inactivation by human serum.

In accordance with an aspect of the present invention, there is provided a vector particle which is resistant to inactivation by human serum. Preferably, the vector particle is a viral vector particle, and more preferably the viral vector particle is a retroviral vector particle.

The envelope portion of retroviruses include a protein known as p15E, and Applicants have found that retroviruses are susceptible to inactivation by human serum as a result of the action of complement protein(s) present in serum on the p15E protein portion of the retrovirus. Applicants have further found that such retroviruses can be made resistant to inactivation by human serum by mutating such p15E protein.

In one embodiment, there is provided a retroviral vector wherein a portion of the DNA encoding p15E protein (shown in the accompanying sequence listing), has been mutated to render the vector particle resistant to inactivation by human serum. The terms "mutated" and "mutation" as used herein mean that the DNA encoding p15E protein has been changed such that at least one but not all of the amino acids of p15E protein have been changed (such changes can include point mutations, deletions, and/or insertions).

p15E protein is a viral protein having 196 amino acid residues. In viruses, sometimes all 196 amino acid residues are present, and other times, amino acid residues 181 to 196 (known as the "r" peptide), are not present, and the resulting protein is the "mature" form of p15E known as p12E. Thus, viruses can contain both the p15E and p12E proteins. p15E protein is anchored in the viral membrane such that amino acid residues 1 to 134 are present on the outside of the virus. Although the present invention is not to be limited to any of the following reasoning, Applicants believe complement proteins may bind to this region whereby such binding leads to inactivation and/or lysis of the retrovirus. In particular, the p15E protein includes two regions, amino acid residues 39 to 61 (sometimes hereinafter referred to as region 1), and amino acid residues 101 to 123 (sometimes hereinafter referred to as region 2), which Applicants believe have an external location in the three-dimensional structure of the p15E protein; i.e., such regions are directly exposed to human serum. Region 2 is a highly conserved region in many retroviruses, even though the amino acid sequences of this region are not identical in all retroviruses. Such regions are complement binding regions. Examples of complement proteins which may bind to the complement binding regions are C1S and C1Q, which bind to regions 1 and 2.

In order to inactivate the retrovirus, complement proteins bind to both region 1 and region 2. Thus, in a preferred embodiment, at least one portion of DNA encoding a complement binding region of p15E protein has been mutated. Such a mutation results in a change of at least one amino acid residue of a complement binding region of p15E protein. The change in at least one amino acid residue of a complement binding region of p15E protein prevents binding of a complement protein to the complement binding region, thereby preventing complement inactivation of the retrovirus. In one embodiment, at least one amino acid residue in both complement binding regions of p15E protein is changed, whereas in another embodiment, at least one amino acid residue in one of the complement binding regions is changed.

It is to be understood, however, that the entire DNA sequence encoding p15E protein cannot be mutated because such a change renders the vectors unsuitable for in vivo use.

In one embodiment, the at least one portion of DNA encoding p15E protein is mutated such that at least one positively charged amino acid residue or negatively charged amino acid residue is changed to an amino acid residue having the opposite charge.

The positively charged amino acids are His, Lys, and Arg.

The negatively charged amino acids are Asp and Glu.

In another embodiment, the at least one portion of DNA encoding p15E protein is mutated such that at least one positively charged amino acid or negatively charged amino acid is changed to a noncharged amino acid.

In one embodiment, the at least one portion of DNA encoding a complement binding region of p15E protein, which is mutated, encodes one or more of amino acid residues 101 to 123 of p15E protein. In one embodiment, the at least one portion of DNA encoding p15E protein is mutated such that amino acid residue 122 is changed.

In one embodiment, the at least one portion of DNA encoding p15E protein is mutated such that at least one of amino acid residues 117 and 122 are changed. Preferably, amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln.

In another embodiment, the at least one portion of DNA encoding p15E protein is mutated such that amino acid residues 104, 105, 109, and 111 are changed. Preferably, amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, and amino acid residue 111 is changed from Arg to Gln.

In another embodiment, the at least one portion of DNA encoding p15E protein is mutated such that amino acid residues 104, 105, 109, 111, 117, and 122 are changed. Preferably, the at least one portion of DNA is mutated such that amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, amino acid residue 111 is changed from Arg to Gln, amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln.

In yet another alternative embodiment, the mutation of DNA encoding p15E protein may be effected by deleting a portion of the p15E gene, and replacing the deleted portion of the p15E gene, with fragment(s) or portion(s) of a gene encoding another viral protein. In one embodiment, one portion of DNA encoding the p15E protein is replaced with a fragment of the gene encoding the p21 protein, which is an HTLV-I transmembrane protein. HTLV-I virus has been found to be resistant to binding by complement proteins and thus HTLV-I is resistant to inactivation by human serum (Hoshino, et al., Nature, Vol. 310, pgs. 324-325 (1984)). Thus, in one embodiment, there is also provided a retroviral vector particle wherein a portion of the p15E protein has been deleted and replaced with a portion of another viral protein, such as a portion of the p21 protein.

p21 protein (as shown in the accompanying sequence listing) is a protein having 176 amino acid residues, and which, in relation to p15E, has significant amino acid sequence homology. In one embodiment, at least amino acid residues 39 to 61, and 101 to 123 are deleted from p15E protein, and replaced with amino acid residues 34 to 56 and 96 to 118 of p21 protein. In one alternative, at least amino acid residues 39 to 123 of p15E protein are deleted and replaced with amino acid residues 34 to 118 of p21 protein.

In another embodiment, amino acid residues 39 to 69 of p15E protein are deleted and replaced with amino acid residues 34 to 64 of p21 protein, and amino acid residues 96 to 123 of p15E protein are deleted and replaced with amino acid residues 91 to 118 of p21 protein.

Vector particles thus generated, and which are resistant to inactivation by human serum, may be engineered such that the vector particles may, when introduced into a patient, travel directly to a target cell or tissue. Thus, in a preferred embodiment, the vector particle further includes a protein which contains a receptor binding region that binds to a receptor of a human target cell, such as, for example, but not limited to, the amphotropic cell receptor.

The retroviral vectors hereinabove described, may be constructed by genetic engineering techniques known to those skilled in the art.

In one embodiment, the retroviral vector may be of the LN series of vectors, as described in Bender, et al., J.Virol., Vol. 61, pgs. 1639-1649 (1987) and Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989).

In another embodiment, the retroviral vector includes a multiple restriction enzyme site, or multiple cloning site.

The multiple cloning site includes at least four cloning, or restriction enzyme sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average size of at least 10,000 base pairs.

In general, such restriction sites, also sometimes hereinafter referred to as "rare" sites, which have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs, contain a CG doublet within their recognition sequence, such doublet appearing particularly infrequently in the mammalian genome. Another measure of rarity or scarcity of a restriction enzyme site in mammals is its representation in mammalian viruses, such as SV40. In general, an enzyme whose recognition sequence is absent in SV40 may be a candidate for being a "rare" mammalian cutter.

Examples of restriction enzyme sites having an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs include, but are not limited to the NotI, SnaBI, SalI, XhoI, ClaI, SacI, EagI, and SmaI sites. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI.

Preferably, the multiple cloning site has a length no greater than about 70 base pairs, and preferably no greater than about 60 base pairs. In general, the multiple restriction enzyme site, or multiple cloning site is located between the 5'LTR and 3'LTR of the retroviral vector. The 5' end of the multiple cloning site is no greater than about 895 base pairs from the 3' end of the 5' LTR, and preferably at least about 375 base pairs from the 3' end of the 5' LTR. The 3' end of the multiple cloning site is no greater than about 40 base pairs from the 5' end of the 3' LTR, and preferably at least 11 base pairs from the 5' end of the 3' LTR.

Such vectors may be engineered from existing retroviral vectors through genetic engineering techniques known in the art such that the resulting retroviral vector includes at least four cloning sites wherein at least two of the cloning sites are selected from the group consisting of the NotI, SnaBI, SalI, and XhoI cloning sites. In a preferred embodiment, the retroviral vector includes each of the NotI, SnaBI, SalI, and XhoI cloning sites.

Such a retroviral vector may serve as part of a cloning system for the transfer of genes to eukaryotic cells. Thus, there may be provided a cloning system for the manipulation of genes in a retroviral vector which includes a retroviral vector including a multiple cloning site of the type hereinabove described, and a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from said shuttle cloning vector to said retroviral vector.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites hereinabove described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC18; etc.

It is also contemplated that within the scope of the present invention that the DNA encoding p15E protein which has been mutated to render a vector particle resistant to inactivation by human serum, may be contained in an expression vehicle other than a retroviral vector. Such expression vehicles include, for example, viral vectors other than retroviral vectors, or any expression plasmid which is capable of being transferred into a cell line which is capable of producing vector particles which include the mutated p15E protein.

Such vectors or expression vehicles which contain DNA encoding a mutated env protein such as the mutated p15E protein hereinabove described, are transferred into a pre-packaging cell line to generate vector particles. In general, the pre-packaging cell line contains the gag and pol proteins of the virus, plus a retroviral vector lacking the structural gag, pol, and env proteins. An example of such a pre-packaging cell line is the GPL pre-packaging cell line which consists of an NIH 3T3 mouse fibroblast cell line which contains an expression plasmid for MoMuLV gag-pol protein as well as the retroviral vector LNL6 (Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989)). It is to be understood, however, that the scope of the present invention is not to be limited to any particular pre-packaging cell line.

Upon transfection of the pre-packaging cell line with an expression vehicle containing DNA encoding a mutated env protein, the pre-packaging cell line will generate vector particles. The vector particles are then tested for complement resistance. The vector particles which are shown to be complement resistant (i.e., not inactivated by human serum), therefore, contain complement resistant envelope proteins encoded by a specific envelope expression vehicle. Such an expression vehicle can then be used, by techniques known to those skilled in the art, to produce a packaging cell line which contains an expression vehicle encoding the retroviral gag and pol proteins, and an expression vehicle containing a gene encoding the mutated env protein (such as, for example, an expression vehicle or expression plasmid containing a mutated p15E protein such as hereinabove described), whereby such packaging cell line may be employed to generate vector particles which are resistant to inactivation by human serum. In particular, a retroviral vector which lacks the structural gag, pol, and env genes, but includes a desired gene of interest, may be transferred into such a packaging cell line. Thus, the packaging cell line may generate vector particles which contain a desired gene(s) of interest, and which are resistant to inactivation by human serum.

The vector particles generated from the packaging cell line will not be inactivated when contacted with human serum; and in addition, such vector particles, when engineered with protein containing a receptor binding region for a human receptor, are targetable, whereby the receptor binding region for a human receptor enables the vector particles to bind to a target cell. Thus, such retroviral vector particles may be directly introduced into the body (e.g., by intravenous, intramuscular, or subcutaneous injection, intranasally, orally, rectally or vaginally), and travel to a desired target cell. Such vector particles, therefore, are useful for the introduction of desired heterologous genes into target cells in vivo as a gene therapy procedure.

Thus, preferably, the vectors of the present invention further include at least one heterologous gene. Heterologous or foreign genes which may be placed into the vector or vector particles include, but are not limited to, genes which encode cytokines or cellular growth factors, such as lymphokines, which are growth factors for lymphocytes. Other examples of foreign genes include, but are not limited to, genes encoding Factor VIII, Factor IX, tumor necrosis factors (TNF's), ADA, ApoE, ApoC, and Protein C.

The vectors of the present invention include one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, pgs. 980-990 (1989), or any other promoter (eg., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The vectors of the present invention may contain regulatory elements, where necessary, to ensure tissue specific expression of the desired heterologous gene(s), and/or to regulate expression of the heterologous gene(s) in response to cellular or metabolic signals.

Although the invention has been described with respect to retroviral vector particles, other viral vector particles (such as, for example, adenovirus and adeno-associated virus particles), or synthetic particles may be constructed wherein a region of the envelope protein in the vector particle may be mutated such that the vector particle becomes resistant to inactivation by human serum, thereby making such vector particles suitable for in vivo administration.

The invention will now be described with respect to the following example; however, the scope of the present invention is not intended to be limited thereby.

Example

Plasmid pCE2 was constructed from pBR322 such that the resulting plasmid pCE2 includes genes encoding the envelope proteins gp70 and p15E. pBR322 (Figure 1) was cut with EcoRI and filled in to destroy the EcoRI site to give pBR322Δ RI. pBR322ΔR1 was then cut with NdeI and filled in to destroy the NdeI site to give pBR322 Δ R Δ N. pBR322Δ R Δ N was digested with HindIII and EcoRV, and cloned into the HindIII/EcoRV fragment was a HindIII/FspI cassette containing the gp70 and p15E genes under the control of a cytomegalovirus (CMV) intermediate early promoter with a polyA (adenine) tail from SV40 (Figure 2) from plasmid pCEE. (Figure 3). The HindIII/FspI cassette obtained from plasmid pCEE contains a CMV intermediate early promoter in which the BalI/SacII (bp 21 to bp 766) was converted to an HindIII/SalI fragment by linker addition; the ecotropic envelope BglII/NheI fragment (bp 5408 to bp 7847 of MoMuLV, encoding gp70 and p15E) was filled and EcoRI linkers were added; and the SV40 poly A signal from BclI to BamHI (bp 2770 to bp 2533) was cloned into a BamHI site (thereby destroying the BclI site). A BglII site was added at the 3' end of the gp70 gene. (This addition does not alter any amino acids). The resulting plasmid is pCE2 (Figure 4.)

To create the mutations in the p15E gene, subcloning is carried out in a different plasmid called pUC-E2. (Figure 6.) This plasmid is pUC18 (Figure 5) with the PvuII fragment removed and replaced with EcoRI linkers. Such was accomplished by digesting pUC18 with PvuII to remove a 322 bp PvuII fragment, and EcoRI linkers were then added. Into the EcoRI site was cloned the MoMuLV ecotropic envelope gene (i.e., the gp70 and p15E genes from pCE2) from the BglII site (5408) to the NheI site (7847), which have been blunted and had EcoRI linkers added. The resulting pUC-E2 plasmid (Figure 6) therefore has unique BglII, SpeI, ClaI, and PvuII sites in and around the p15E gene.

PCR primers are then synthesized to encode the following mutations in the p15E protein (using pCE2 as a template):

1. Amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln;
2. Amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, and amino acid residue 111 is changed from Arg to Gln; and
3. Amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, amino acid residue 111 is changed from Arg to Gln, amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln.

Each PCR product is digested with SpeI and PvuII, and cloned into pUC-E2 at the unique SpeI and PvuII sites. The resulting plasmids are then sequenced to confirm the point mutations.

DNA fragments bearing these mutations are then subcloned into the expression plasmid pCE2. pCE2 is digested with EcoRI and the envelope DNA fragment is removed and replaced with the EcoRI envelope fragment from the pUC-E2 plasmids. The resulting pCE2 plasmids are then checked for orientation of the EcoRI fragment and sequenced again (only at the cloning site junctions and at the regions bearing the point mutations) to confirm the presence of the newly created mutated p15E genes. The resulting expression plasmids are identified as follows:

pCR68 - includes mutations in which amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln;

pCR69 - includes mutations in which amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, and amino acid residue 111 is changed from Arg to Gln; and

pCR70 - includes mutations in which amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, amino acid residue 111 is changed from Arg to Gln, amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln.

Plasmids pCR68, pCR69, and pCR70 are transfected separately into the GPL pre-packaging cell line. The GPL pre-packaging cell line consists of an NIH 3T3 mouse fibroblast cell line which contains an expression plasmid for MoMuLV gag-pol protein as well as the retroviral vector LNL6 (Miller, et al., 1989). Upon transfection with pCR68, pCR69, or pCR70, the GPL packaging cell line produces vector particles.

Transiently expressed vector particles are collected with cell supernatant at 48-72 hrs. post-transfection.

The vector particles generated as hereinabove described may then be assayed for vector titer by techniques known to those skilled in the art. The vector particles may also be collected in viral supernatant and concentrated, if necessary, according to procedures known to those skilled in the art in order to employ such vector particles in assays or in therapeutic procedures.

Advantages of the present invention include the ability to introduce vector particles directly into a human patient whereby the vector particle is not lysed or inactivated by human serum upon such introduction. Thus, the vector particles of the present invention enable one to deliver desired genes to a patient in vivo. Such vector particles may also be engineered such that they are "targetable", as well as injectable, thereby enabling the vector particles to travel directly to a target cell or tissue without being lysed or inactivated by human serum.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Vector Particles Resistant to Inactivation by Human Serum

(iii) NUMBER OF SEQUENCES: 2

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(vi) CURRENT APPLICATION DATA:
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- (B) REGISTRATION NUMBER: 24,025
- (C) REFERENCE/DOCKET NUMBER: 271010-73

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-994-1700
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(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 196 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(ix) FEATURE:
(A) NAME/KEY: p15E protein.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Glu Pro Val Ser Leu Thr Leu Ala Leu Leu
                5                   10
Leu Gly Gly Leu Thr Met Gly Gly Ile Ala
                15                  20
Ala Gly Ile Gly Thr Gly Thr Thr Ala Leu
                25                  30
Met Ala Thr Gln Gln Phe Gln Gln Leu Gln
                35                  40
Ala Ala Val Gln Asp Asp Leu Arg Glu Val
                45                  50
Glu Lys Ser Ile Ser Asn Leu Glu Lys Ser
                55                  60
Leu Thr Ser Leu Ser Glu Val Val Leu Gln
                65                  70
Asn Arg Arg Gly Leu Asp Leu Leu Phe Leu
                75                  80
Lys Glu Gly Gly Leu Cys Ala Ala Leu Lys
                85                  90
Glu Glu Cys Cys Phe Tyr Ala Asp His Thr
                95                  100
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Gly Leu Val Arg Asp Ser Met Ala Lys Leu
                105                 110
Arg Glu Arg Leu Asn Gln Arg Gln Lys Leu
                115                 120
Phe Glu Ser Thr Gln Gly Trp Phe Glu Gly
                125                 130
Leu Phe Asn Arg Ser Pro Trp Phe Thr Thr
                135                 140
Leu Ile Ser Thr Ile Met Gly Pro Leu Ile
                145                 150
Val Leu Leu Met Ile Leu Leu Phe Gly Pro
                155                 160
Cys Ile Leu Asn Arg Leu Val Gln Phe Val
                165                 170
Lys Asp Arg Ile Ser Val Val Gln Ala Leu
                175                 180
Val Leu Thr Gln Gln Tyr His Gln Leu Lys
                185                 190
Pro Ile Glu Tyr Glu Pro
                195
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE
(A) NAME/KEY: p21 protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Val Pro Val Ala Val Trp Leu Val Ser
5 10
Ala Leu Ala Met Gly Ala Gly Val Ala Gly
15 20
Arg Ile Thr Gly Ser Met Ser Leu Ala Ser
25 30
Gly Lys Ser Leu Leu His Glu Val Asp Lys
35 40
Asp Ile Ser Gln Leu Thr Gln Ala Ile Val
45 50
Lys Asn His Lys Asn Leu Leu Lys Ile Ala
55 60
Gln Tyr Ala Ala Gln Asn Arg Arg Gly Leu
65 70
Asp Leu Leu Phe Trp Glu Gln Gly Gly Leu
75 80
Cys Lys Ala Leu Gln Glu Gln Cys Cys Phe
85 90
Leu Asn Ile Thr Asn Ser His Val Ser Ile
95 100
Leu Gln Glu Arg Pro Pro Leu Glu Asn Arg
105 110
Val Leu Thr Gly Trp Gly Leu Asn Trp Asp
115 120
Leu Gly Leu Ser Gln Trp Ala Arg Glu Ala
125 130
Leu Gln Thr Gly Ile Thr Leu Val Ala Leu
135 140
Leu Leu Leu Val Ile Leu Ala Gly Pro Cys
145 150
Ile Leu Arg Gln Leu Arg His Leu Pro Ser
155 160

```
Arg Val Arg Tyr Pro His Tyr Ser Leu Ile
                165                 170
Asn Pro Glu Ser Ser Leu
                175
```

WHAT IS CLAIMED IS:

1. A retroviral vector particle, said vector particle being resistant to inactivation by human serum.

2. The vector particle of Claim 1 wherein said vector particle includes p15E protein, and wherein a portion but not all of the p15E protein has been mutated to render the vector particles resistant to inactivation by human serum.

3. The vector particle of Claim 2 wherein a portion of the p15E protein has been mutated such that at least one amino acid of p15E protein has been changed.

4. The vector particle of Claim 3 wherein at least one portion of a complement binding region of p15E protein has been mutated.

5. The vector particle of Claim 4 wherein said complement binding region is selected from the group consisting of amino acid residues 39 to 61 and 101 to 123 of p15E protein.

6. The vector particle of Claim 5 wherein said complement binding region is amino acid residues 101 to 123 of p15E protein.

7. The vector particle of Claim 6 wherein p15E protein is mutated such that amino acid residue 122 of p15E protein is changed.

8. The vector particle of Claim 6 wherein p15E protein is mutated such that amino acid residues 117 and 122 are changed.

9. The vector particle of Claim 8 wherein p15E protein is mutated such that amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln.

10. The vector particle of Claim 6 wherein p15E protein is mutated such that amino acid residues 104, 105, 109, and 111 are changed.

11. The vector particle of Claim 10 wherein p15E protein is mutated such that amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, and amino acid residue 111 is changed from Arg to Gln.

12. The vector particle of Claim 6 wherein p15E protein is mutated such that amino acid residues 104, 105, 109, 111, 117, and 122 are changed.

13. The vector particle of Claim 12 wherein p15E protein is mutated such that amino acid residue 104 is changed from Arg to His, amino acid residue 105 is changed from Asp to Asn, amino acid residue 109 is changed from Lys to Gln, amino acid residue 111 is changed from Arg to Gln, amino acid residue 117 is changed from Arg to Glu, and amino acid residue 122 is changed from Glu to Gln.

14. The vector particle of Claim 5 wherein p15E protein is mutated such that at least one positively or negatively charged amino acid is changed to an amino acid having the opposite charge.

15. The vector particle of Claim 5 wherein p15E protein is mutated such that at least one positively charged or negatively charged amino acid is changed to a non-charged amino acid.

16. The vector particle of Claim 1 wherein said vector particle further includes a protein containing a receptor binding region that binds to a receptor of a human target cell.

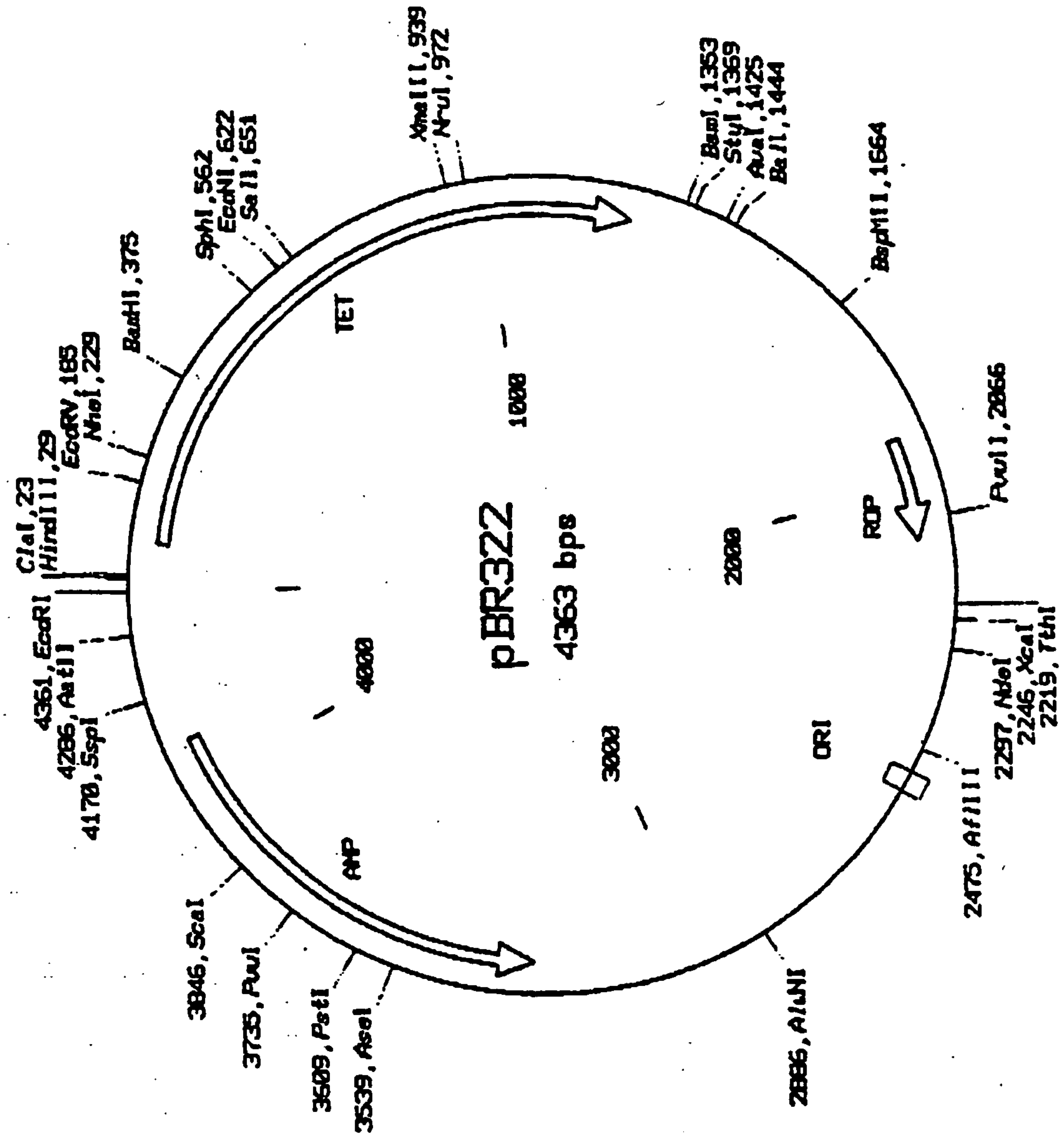
17. The vector particle of Claim 16 and further containing a heterologous gene.

18. Eukaryotic cells transformed with the vector particles of Claim 17.

19. An expression vehicle including DNA encoding p15E protein, wherein said DNA encoding p15E protein is mutated such that when said mutated p15E protein encoded by said DNA is included in a vector particle, said vector particle is resistant to inactivation by human serum.

20. A packaging cell line including the expression vehicle of Claim 19.

21. A viral vector particle, said viral vector particle being resistant to inactivation by human serum.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EcoRV,185
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BamHI,375
SphI,562
EcoNI,622
SalI,651
XmaIII,939
NruI,972
BsmI,1353
StyI,1369
AvaI,1425
BalI,1444
BspMII,1664
PvuII,2066
2297,NdeI
2246,XcaI
2219,TthI
2475,AflIII
2886,AlwNI
3539,AseI
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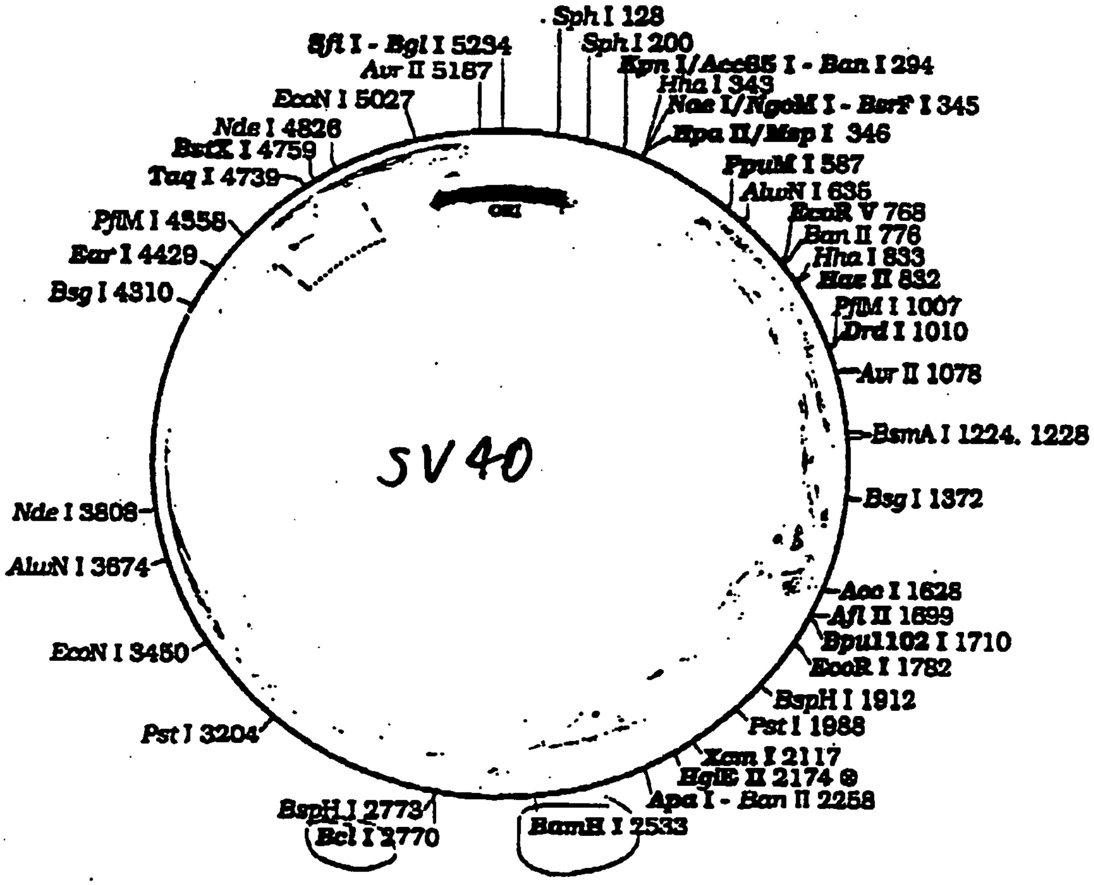
4361,EcoRI

Fig.1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Sph I 200
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Nae I/NgoM I - BsrF I 345
Hpa II/Msp I 346
PpuM I 587
AluN I 636
EcoR V 768
Ban II 776
Hha I 833
Hae II 832
PflM I 1007
Drd I 1010
Avr II 1078
BsmA I 1224. 1228
Bsg I 1372
Acc I 1628
Afl II 1699
Bpu1102 I 1710
EcoR I 1782
BspH I 1912
Pst I 1988
Xcm I 2117
HgiE II 2174
Apa I - Ban II 2258
BamH I 2533
BspH I 2773
Bcl I 2770
Pst I 3204
EcoN I 3450
AluN I 3674
Nde I 3808
Bsg I 4310
Ear I 4429
PflM I 4558
Taq I 4739
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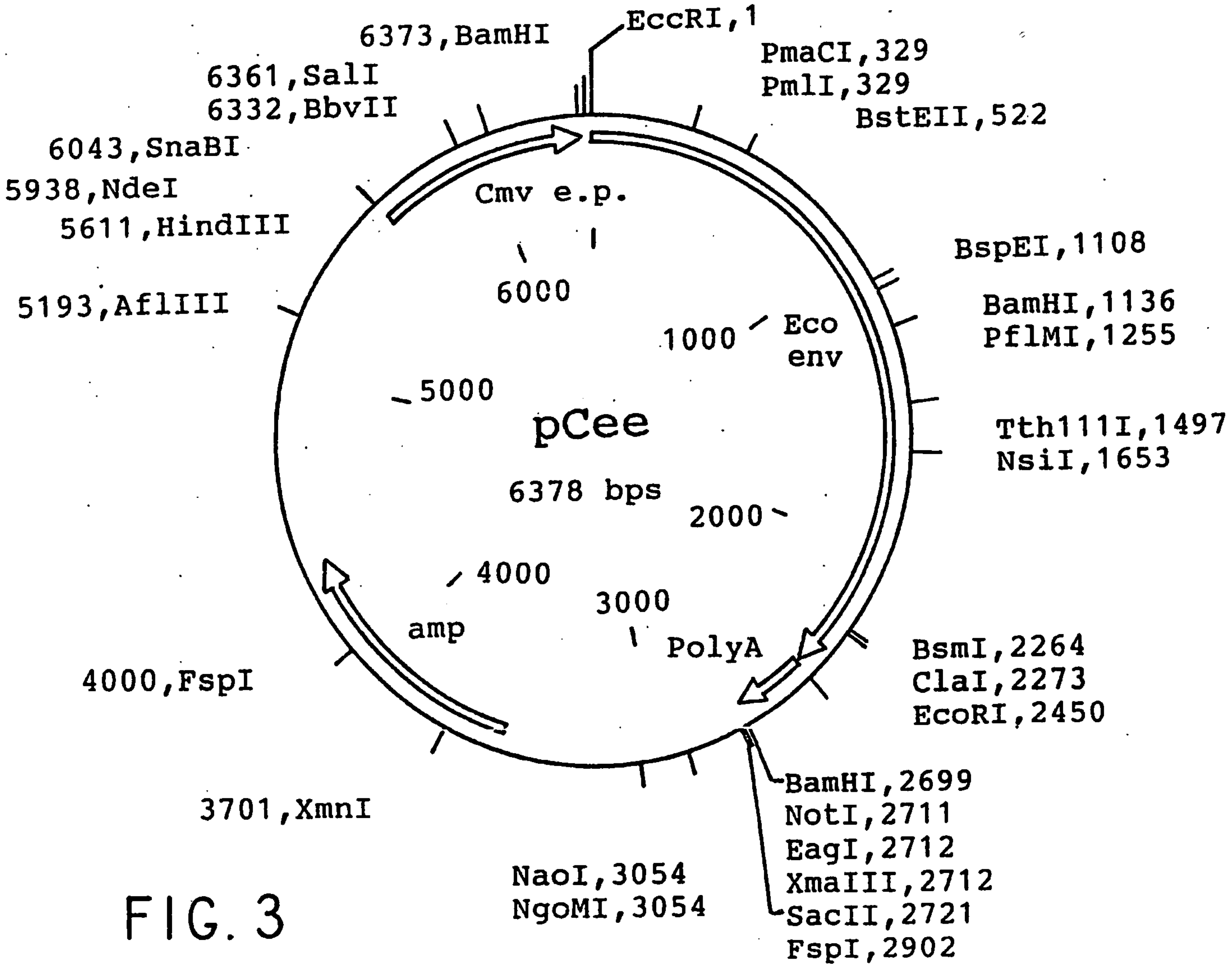
EcoN I 5027
Avr II 5187
Sfi I - Bgl I 5234
ori
SV40

Fig. 2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BsmI,2264
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NotI,2711
EagI,2712
XmaIII,2712
SacII,2721
FspI,2902
NaoI,3054
NgoMI,3054
3701,XmnI
4000,FspI
5193,AflIII
5611,HindIII
5938,NdeI
6043,SnaBI
6332,BbvII
6361,SalI
6373,BamHI
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Eco
env
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amp
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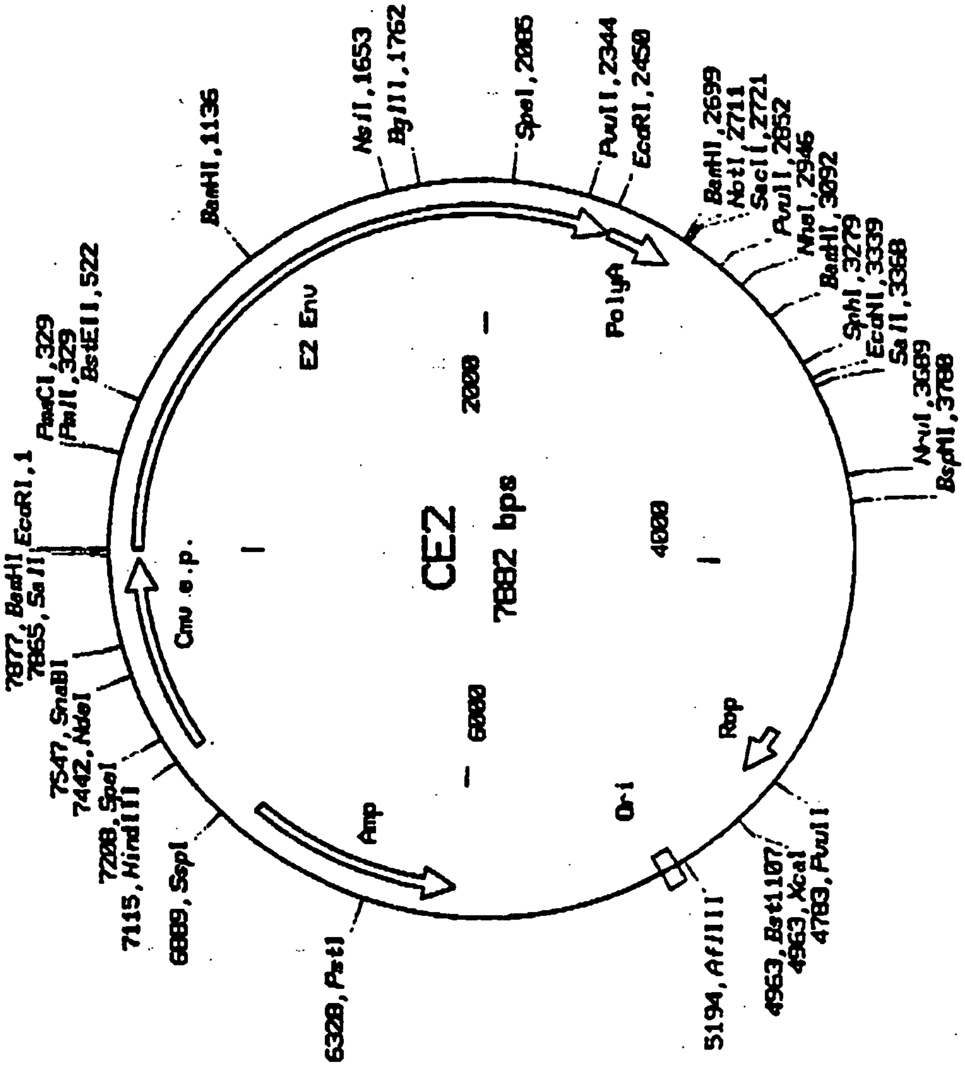
4000
5000
6000

FIG. 3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4000
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E2 Env
PolyA
Cmv e.p.
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Rop
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PmlI,329
BstEII,522
BamHI,1136
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BglII,1762
SpeI,2085
PvuII,2344
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BamHI,2699
NotI,2711
SacII,2721
PvuI,2852
NheI,2946
BamHI,3092
SphI,3279
EcoNI,3339
SalI,3368
NruI,3689
BspMI,3708
4783,PvuII
4963,XcaI
4963,Bst1107
5194,AflIII
6328,PstI
6889,SspI
7115,HindIII
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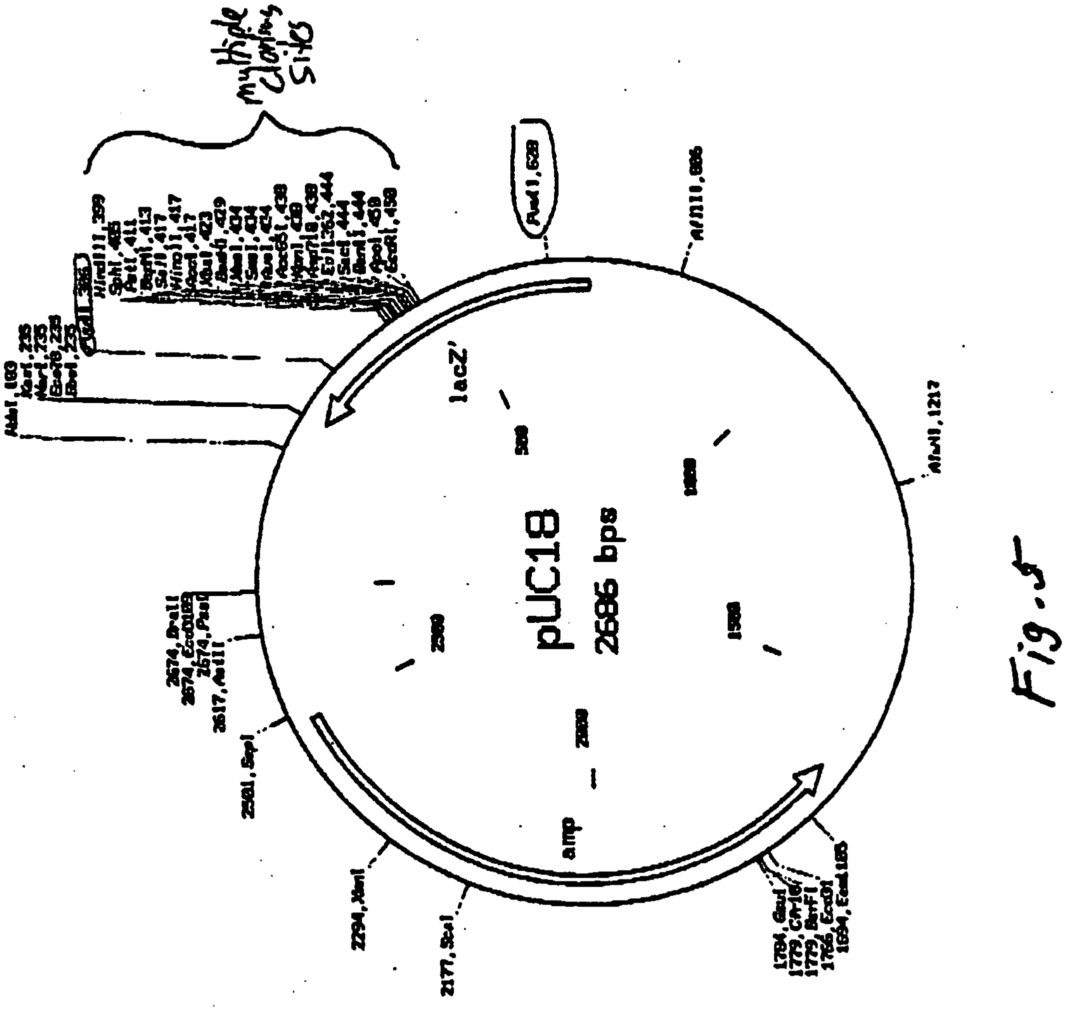
7442,NdeI
7547,SnaBI
7865,SalI
7877,BamHI

Fig. 4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AccI,417
XbaI,423
BamHI,429
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SmaI,434
AvaI,434
Acc65I,438
KpnI,438
Asp718,438
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SacI,444
BanII,444
ApoI,450
EcoRI,450
PvuII,628
AflIII,806
AlwNI,1217
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1000
1500
2000
2500
pUC18
2686 bps
amp
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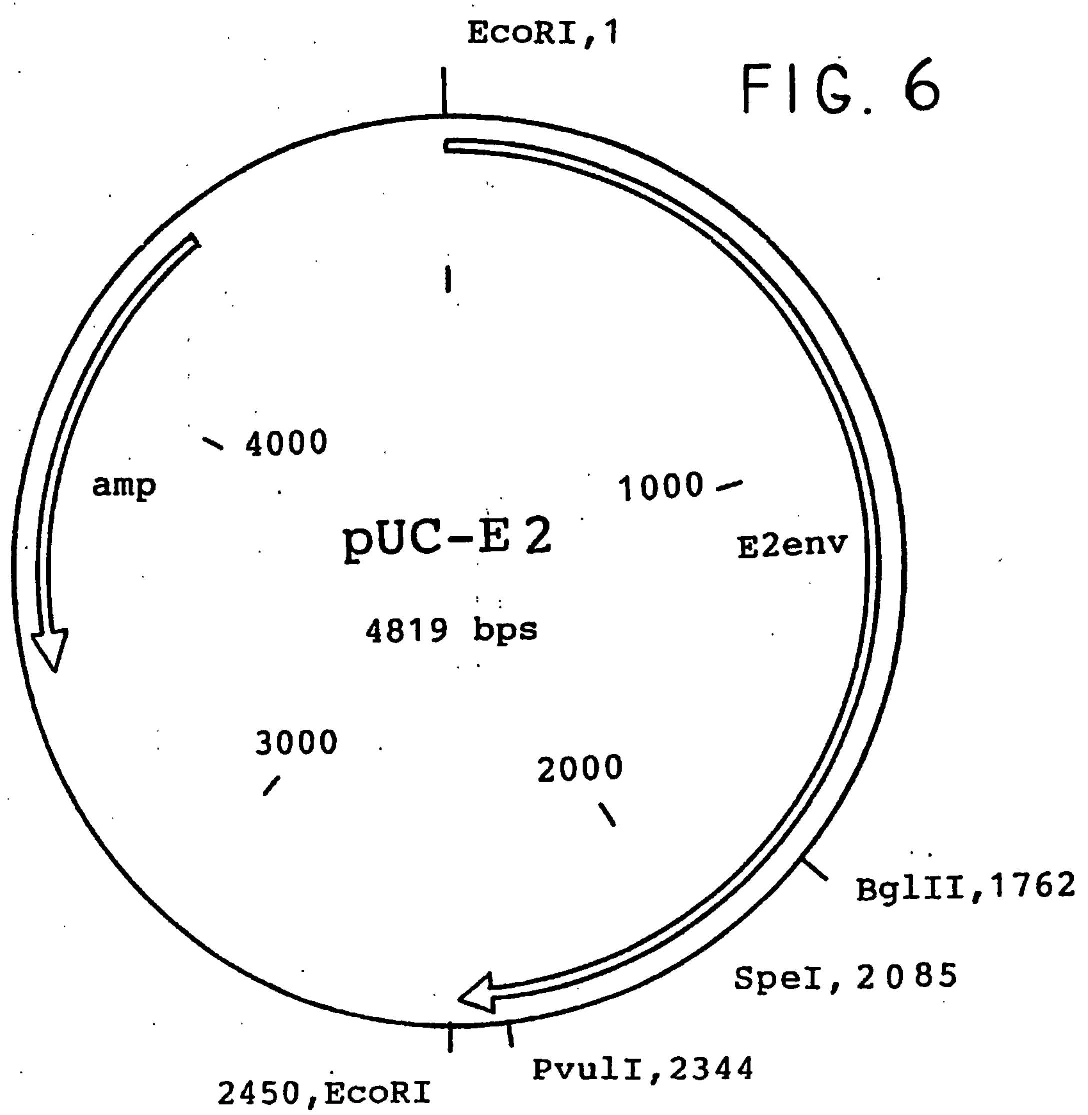
1779,Cfr10I
1779,BsrFI
1766,Eco31I
1694,Eam1105I
2177,ScaI
2294,XmnI
2501,SspI
2617,AatII
2674,DraII
2674,EcoO109I
2674,PssI

Fig. 5

FIG. 6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pUC-E2
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3000
4000
amp
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SpeI,2085
PvuII,2344
2450,EcoRI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04706

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 21/06; C12N 15/00, 7/00, 5/00; C07K 3/00, 13/00, 15/00
US CL :435/69.1, 172.1, 235.1, 240.2, 320.1; 536/23.1, 23.72; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.1, 235.1, 240.2, 320.1; 536/23.1, 23.72; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medicine, Medline
search terms: retrovirus, p15e, inactivate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Virology, Volume 61, No. 5, issued May 1987, M. A. Bender et al., "Evidence that the Packaging Signal of Moloney Murine Leukemia Virus Extends into the gag Region", pages 1639-1646, see whole article, particularly Fig. 1.	1-21
A	Nature, Volume 310, issued 26 July 1984, H. Hoshino et al., "Human T-Cell Leukemia Virus is not Lysed by Human Serum", pages 324-325, see whole article.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 JULY 1993	Date of mailing of the international search report 14 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DAVID GUZO
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{5} : **C12N 15/86, 15/12, A61K 48/00**	A3	**(11) International Publication Number:** **WO 94/12649** **(43) International Publication Date:** 9 June 1994 (09.06.94)

(21) International Application Number: PCT/US93/11667

(22) International Filing Date: 2 December 1993 (02.12.93)

(30) Priority Data:

07/985,478	3 December 1992 (03.12.92)	US
08/130,682	1 October 1993 (01.10.93)	US
08/136,742	13 October 1993 (13.10.93)	US

(71) Applicant: GENZYME CORPORATION [US/US]; One Kendall Square, Cambridge, MA 02139 (US).

(72) Inventors: GREGORY, Richard, J.; 4789 Gateshead Road, Carlsbad, CA 92008 (US). ARMENTANO, Donna; 33 Carver Road, Watertown, MA 02172 (US). COUTURE, Larry, A.; 67 Circle Drive, Framingham, MA 01701 (US). SMITH, Alan, E.; 88 Cleveland Road, Wellesley, MA 02181 (US).

(74) Agents: HANLEY, Elizabeth, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

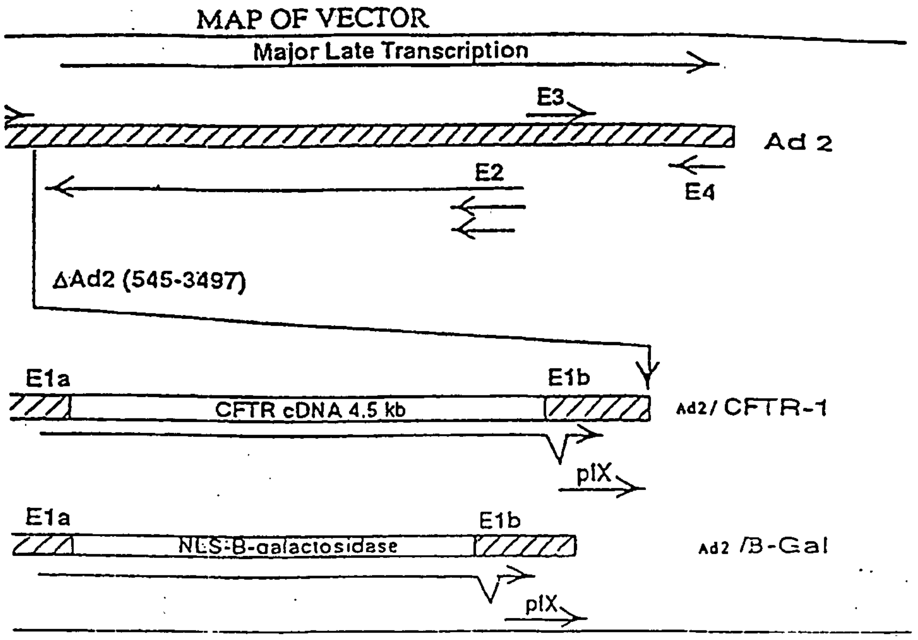
(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report: 10 November 1994 (10.11.94)

(54) Title: GENE THERAPY FOR CYSTIC FIBROSIS

(57) Abstract



Gene Therapy vectors, which are especially useful for cystic fibrosis, and methods for using the vectors are disclosed. In preferred embodiments, the vectors are adenovirus-based. Advantages of adenovirus-based vectors for gene therapy are that they appear to be relatively safe and can be manipulated to encode the desired gene product and at the same time are inactivated in terms of their ability to replicate in a normal lytic viral life cycle. Additionally, adenovirus has a natural tropism for airway epithelia. Therefore, adenovirus-based vectors are particularly preferred for respiratory gene therapy applications such as gene therapy for cystic fibrosis. In one embodiment, the adenovirus-based gene therapy vector comprises an adenovirus 2 serotype genome in which the Ela and Elb regions of the genome, which are involved in early stages of viral replication have been deleted and replaced by genetic material of interest (e.g., DNA encoding the cystic fibrosis transmembrane regulator protein). In another embodiment, the adenovirus-based therapy vector is a pseudo-adenovirus (PAV). PAVs contain no potentially harmful viral genes, have a theoretical capacity for foreign material of nearly 36 kb, may be produced in reasonably high titers and maintain the tropism of the parent adenovirus for dividing and non-dividing human target cell types.

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CZ	Czech Republic
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GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
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LI	Liechtenstein
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MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
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INT ATIONAL SEARCH REPORT

In d Application No
PCT/US 93/11667

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/86 C12N15/12 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELL., vol.75, no.2, 22 October 1993, CAMBRIDGE, NA US pages 207 - 216 ZABNER, J. ET AL. 'Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with Cystic Fibrosis' see the whole document ---	1-5,8,18
P,X	FR,A,2 688 514 (CNRS) 17 September 1993 see page 2, line 25 - page 3, line 5 --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

5

Date of the actual completion of the international search 30 May 1994	Date of mailing of the international search report -4 -10- 1994
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer CHAMBONNET, F

In[illegible]l Application No
PCT/US 93/11667

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH., vol.11, no.24, 1983, ARLINGTON, VIRGINIA US pages 8735 - 8745 SASSONE-CORSI, P. ET AL. 'Far upstream sequences are required for efficient transcription from the adenovirus-2 E1A transcription unit' see the whole document ---	1
X	EP,A,0 185 573 (INSERM) 25 June 1986 see the whole document ---	1
Y	CELL., vol.68, no.1, 10 January 1992, CAMBRIDGE, NA US pages 143 - 155 ROSENFELD, M.A. ET AL. 'In vivo transfer of the human Cystic Fibrosis Transmembrane Conductance Regulator gene to the airway epithelium' see the whole document ---	1-5,8,18
Y	EP,A,0 446 017 (GENZYME CORPORATION) 11 September 1991 cited in the application see page 21 - page 23; claims 21,28-30,65,67 -----	1-5,8,18

INTERNATIONAL SEARCH REPORT

....onal application No.
PCT/US93/11667

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

   Remark : Although claims 18,24,25 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   Obscurities : claim 6 refers to "sequens shown in figure 17". However "figure 17 shows an example of UV fluorescence from an agarose electrophoresis (p7, 1.1)"

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1-5,7,8,18 (completely) ; 11,14,24,25 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

LACK OF UNITY OF INVENTION

1. Claims 1-5,7,8,18 (completely); 11,14,24,25 (partially):
   Adenovirus-2 based vectors deleted for E1a and E1b genes

2. Claims 9,10,12,13,15,16 (completely); 11,14,22-25 (partially):
   Adenoviral vectors deleted for all E4 open reading frames except 6 or 3

3. Claims 17,19-21 (completely); 22,23 (partially):
   Gene therapy for Cystic Fibrosis by administering to the pulmonary airways of a patient a vector encoding CFTR gene

IN[illegible]NATIONAL SEARCH REPORT

[illegible]ormation on patent family members

In[illegible]l Application No
PCT/US 93/11667

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A-2688514	17-09-93	AU-B- 3757093	21-10-93
		CA-A- 2102302	17-09-93
		EP-A- 0593755	27-04-94
		WO-A- 9319191	30-09-93
EP-A-0185573	25-06-86	FR-A- 2573436	23-05-86
		CA-A- 1266627	13-03-90
		DE-A- 3586092	25-06-92
		JP-A- 61158795	18-07-86
EP-A-0446017	11-09-91	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)